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# Ejaculate evolution in external fertilizers: Influenced by sperm competition or sperm limitation?

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**The evolution of sperm quality and quantity is shaped by various selective processes, with sperm competition generally considered the primary selective agent. Particularly in external fertilizers, however, sperm limitation through gamete dispersal can also influence gamete investments, but empirical data examining this effect are limited. Here, we studied the relative importance of sperm competition and the spawning conditions in explaining the macroevolutionary patterns of sperm size and number within two taxa with external fertilization but differences in their reproductive biology. In frogs, sperm swim slowly but for up to hours as they penetrate the gelatinous egg coating, whereas fish sperm typically swim fast, are very short-lived (seconds to minutes), and often face a relatively higher risk of being moved away from the ova by currents. Our phylogenetic models and path analyses revealed different trajectories of ejaculate evolution in these two taxa. Sperm size and number responded primarily to variation in sperm competition in the anurans, but more strongly to egg number and water turbulence in the fishes. Whereas the results across anurans align with the general expectation that sexual selection is the main driver of ejaculate evolution, our findings across the fishes suggest that sperm limitation has been underappreciated.**

**KEY WORDS:** Anurans, fishes, reproductive investment, sperm number, sperm length, sperm size–number trade-off.

Female multiple mating, causing sperm of different males to compete for fertilization, is considered one of the major drivers of the rapid and diversifying evolution of ejaculate traits (Birkhead and Møller 1998; Birkhead et al. 2009). In principle, selection should favor any ejaculate trait enhancing competitive fertilization success, and positive relationships between ejaculate traits and indices of the strength of sexual selection are indeed widely reported (reviewed in Snook 2005; Pizzari and Parker 2009; Simmons and Fitzpatrick 2012; Fitzpatrick and Lüpold 2014). However, different ejaculate components are unlikely to evolve independently of one another and thus should not be examined in isolation (Gómez Montoto et al. 2011; Immler et al. 2011; Lüpold 2013). A good example of nonindependent evolution is that of sperm size and sperm number, a trade-off that has the strongest theoretical foundation (e.g., Parker 1993; Parker and Begon 1993; Parker et al. 2010).

Early sperm competition models focused primarily on sperm numbers and predicted a competitive advantage for males transferring more sperm than their competitors (Parker 1982, 1993). Assuming limited resources available for sperm production overall, these models also suggested that selection should favor large numbers of tiny sperm, a trade-off that may be further enhanced by spatial constraints within the testes (Pitnick 1996; Lüpold et al. 2009c). However, mounting empirical evidence for positive selection on sperm size complicates these traditional models (Gage 1994; Briskie et al. 1997; Byrne et al. 2003; Fitzpatrick et al. 2009; Lüpold et al. 2009b; Tourmente et al. 2011), particularly if

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selection on sperm size limits sperm quantity to very few sperm per egg and so greatly reduces the male reproductive potential and, in theory, also the intensity of sexual selection (Bjork and Pitnick 2006; Lüpold et al. 2016).

More recent sperm competition models have addressed the conditions under which selection may favor sperm size over sperm number, and when it might favor the reverse (Parker et al. 2010). Using Parker et al.'s (2010) terminology, these models predict intense sperm competition should always select for greater sperm investment overall (i.e., product of sperm size, $m^*$, and sperm number, $s^*$: $m^*s^*$). However, when reaching the capacity of sperm production, the density of sperm around the fertilization site and the mechanism of sperm competition will determine which of the two ejaculate traits should be favored. Stronger selection on sperm size (i.e., increasing $m^*/s^*$) is expected when sperm competition is confined to a relatively tight fertilization site leading to direct interactions between sperm and often sperm displacement from the female reproductive tract (e.g., small insects; Miller and Pitnick 2002; Lüpold et al. 2012; Manier et al. 2013). However, relatively stronger selection on sperm number (i.e., decreasing $m^*/s^*$) is predicted for raffle-like sperm competition and relatively low sperm density, for example when sperm are diluted within a relatively large female reproductive tract (e.g., in larger-bodied organisms; Parker et al. 2010). Recent comparative analyses across species with considerable female size variation empirically support both these predictions (Immler et al. 2011; Lüpold and Fitzpatrick 2015).

As in internal fertilizers with no significant spatial constraints on the processes of raffle-like sperm competition, intensifying selection should also favor sperm number over sperm size in external fertilizers, but to our knowledge there is currently no direct formal test of this prediction. Rather, external fertilizers tend to be assumed to have shorter sperm than internal fertilizers (Franzén 1970; Stockley et al. 1996; Pitnick et al. 2009), and broadcast spawners to invest particularly intensely in sperm quantity (Parker 2016). Yet, positive relationships between sperm length and sperm competition levels have been reported for both anurans (Byrne et al. 2003; Zeng et al. 2014) and fishes (Balshine et al. 2001; Fitzpatrick et al. 2009; but see Stockley et al. 1997), and the painted frog (*Discoglossus pictus*) produces the longest vertebrate sperm examined despite external fertilization (2.5 mm; Pitnick et al. 2009).

Furthermore, the theoretical framework for external fertilizers seems to deviate to some extent from internal fertilizers in both assumptions and predictions. For example, compared to internal fertilizers, in which selection on sperm survival should act from insemination to fertilization (Parker 1993), the simultaneous gamete release between the sexes in most external fertilizers should shift such selection entirely to the fertilization process itself (Ball and Parker 1996). Assuming the number of unfertilized eggs declines at a rate approximately proportional to the density of sperm still alive at any given time, Ball and Parker (1996, 1997) also predicted that the benefits of longer sperm survival should decrease with intensifying sperm competition due to an accelerating decline in fertilizable ova. In other words, males enhance their competitive fertilization success by maximizing the product of the number and swimming speed of their sperm rather than that of sperm number, swimming speed, and longevity. This difference results from the fact that faster sperm swimming can be achieved by a relatively longer sperm tail (provided the necessary energetics) as documented in diverse taxa (Gomendio and Roldan 2008; Fitzpatrick et al. 2009; Lüpold et al. 2009a; Tourmente et al. 2011), but longer sperm tails should also exhaust the available energy faster, resulting in a trade-off between sperm speed and longevity (Ball and Parker 1997).

In addition to sperm competition, optimal ejaculate investment in external fertilizers may further be influenced by egg size and number, or by the spawning environment (e.g., Pennington 1985; Crean and Marshall 2008). For example, larger eggs may increase the probability of sperm–egg encounters under sperm limitation (Levitan 1993, 2006; Rahman and Uehara 2004; Macfarlane et al. 2009), but they may also require sperm with a longer flagellum to generate greater propulsive force for penetration if larger eggs have a thicker vestment (Katz and Drobnis 1990; Byrne et al. 2003). Likewise, greater egg numbers may select for relatively more sperm, for example if unfertilized ova disperse over a larger volume of water and so require a larger and denser cloud of sperm to reach these eggs efficiently, given each sperm can only cover a very short distance as in most fishes (Pennington 1985; Denny and Shibata 1989; Shapiro et al. 1994; Stockley et al. 1996). Finally, the competition among sperm and their probability of encountering an unfertilized egg may also depend on the spawning conditions. For example, gametes released into a nest may be better protected against water currents than those spawned into the open water, and the foam nests of some anuran species may further reduce sperm loss, thereby enhancing sperm survival and fertilization efficiency (Byrne et al. 2002; Edwards et al. 2004) but, in turn, possibly also intensifying sperm competition (Roberts and Byrne 2011).

Here, we examined the macroevolutionary variation in both sperm size and number in response to sperm competition and spawning conditions in externally fertilizing frogs and fishes. In both taxa, comparative studies have documented positive effects of sperm competition on sperm length (Balshine et al. 2001; Byrne et al. 2003; Fitzpatrick et al. 2009; Zeng et al. 2014), but to our knowledge, how sperm size and number vary relative to one another in the context of Parker et al.'s (2010) gamete investment models has not been explored in either taxon. Joint examination of these ejaculate traits, however, is critical due to their nonin-

dependent evolution. Further, Parker et al.'s (2010) models have drawn attention to sperm dilution as a driver of ejaculate evolution beyond postcopulatory sexual selection. With external fertilizers releasing their gametes into the environment, the effects of sperm competition on male gametes should be studied in the context of differential risks of sperm limitation and other selective pressures. Consequently, we examined within each taxon the relative importance of these different selection pressures on the joint evolution of sperm size and number. For a broader understanding of the selective processes and constraints on, and the likely adaptive significance of, male gamete investments, we additionally linked variation in sperm length to sperm function.

At face value, externally fertilizing anurans and fishes should be exposed to similar selective pressures, such as raffle-like sperm competition favoring sperm number over sperm size (Parker et al. 2010). Yet, both taxa differ considerably in important aspects of their reproductive biology. For example, most anurans release gametes during amplexus and so males deposit their sperm on, or very close to, the gelatinous egg coat that has to be infiltrated for fertilization, or they spawn into foam nests (Roberts and Byrne 2011). Hence, anuran sperm are far less likely to compete in a purely aquatic environment than other external fertilizers (Browne et al. 2015). These conditions may lower the risk of sperm dispersal by water currents but intensify selection on the ability of sperm to penetrate the nest foam (where present) and egg capsules, which themselves can vary considerably in their physical properties among species (e.g., Anstis 2013). Further, anuran sperm generally swim slowly but for extended periods (minutes to hours; Browne et al. 2015), with slower but longer-lived sperm having a fertilization advantage in at least some species (Dziminski et al. 2009). In contrast, externally fertilizing fishes always release their sperm into water, where these need to locate eggs and enter their single micropyle (Browne et al. 2015). The spawning location, and thus the likelihood of currents carrying sperm away from the eggs, varies widely across fish species, from egg deposition into protected nests to broadcast spawning into the open water (Balon 1975). The risk of gamete dispersal might be particularly important in species spawning in turbulent rather than stagnant water.

Although our study focused primarily on understanding selection on ejaculates within each of the two taxa, the reproductive differences between them can also help disentangle the relative importance of different modes of selection and ultimately the trajectory of ejaculate evolution under varying spawning conditions. We predicted male investment in ejaculates to covary with sperm competition and sperm limitation levels in both taxa, but sperm dilution should play a relatively more important role in the fishes (strictly aquatic spawning) than in the frogs (generally more precise egg deposition and sperm release into egg jelly or foam).

# Material and Methods

## DATA COLLECTION

For frogs, we compiled data on body mass, testes mass, and sperm morphology (including head, tail and total length) of 130 species, and quantified sperm number in 25 of these species (Suppl. Data File S1). Unless data were taken from the literature, we collected sexually mature males of each species by hand in China at night during the breeding seasons 2009 to 2017. We kept males individually at room temperature in wire-netting rectangular containers ($L \times W \times H = 20 \times 10 \times 15$ cm) placed inside a tank ($90 \times 40 \times 40$ cm) with water to a depth of 10 cm. We weighed all individuals to the nearest 0.1 mg using an electronic balance before sacrificing them by single- or double-pithing (Jin et al. 2016b; Liao et al. 2016) and dissecting their testes.

To obtain sperm quantity data for 24 species (in addition to *Crinia georgiana* from a previous report based on the same general protocol: Hettyey and Roberts 2007), we sterilized and weighed a culture vial ($w_1$) to the nearest 0.1 mg using an electronic balance. After weighing the testes to the nearest 0.1 mg, we immediately crushed them and released sperm into reverse-filtered tap water. We dissolved the sperm suspension and then weighed the culture vial again ($w_2$), transferred 100 μL of sperm suspension to a glass hemocytometer using a pipette, covered the sample with a glass coverslip and counted the sperm heads ($n$) within each of five 1-mm$^2$ areas. Assuming that 1 mL sperm suspension weighed 1 g, we calculated the total number of sperm as $s = 5n \times 10^4 \times (w_2 - w_1)$ (Jin et al. 2016a). Despite relatively few samples available per species ($N = 2-6$), sperm counts varied nearly 3000-fold across species, and species identity explained 93% of the variance ($F_{23,78} = 82.64$, $P < 0.0001$). Clearly, sperm released from macerated testes may not directly reflect sperm contained in natural ejaculates. Yet, in *C. georgiana*, in which different techniques have been employed, Hettyey and Roberts' (2007) testicular sperm counts (mean ± SE = $2.88 \pm 0.42 \times 10^7$ sperm) are comparable to Byrne's (2004) sperm counts from ejaculates collected during matings under laboratory conditions ($2.27 \pm 0.58 \times 10^7$), although they slightly overestimate ejaculates collected in the field ($1.85 \pm 0.22 \times 10^7$; Byrne 2004). We thus assumed that differences between testicular and ejaculated sperm within males were relatively small compared to the multiple orders of magnitude in sperm numbers across species.

For fish, we collected all data from the literature for 57 externally fertilizing freshwater species (Suppl. Data File S1). We restricted our dataset to freshwater fishes due to the small number of marine species with available data and different selective pressures on sperm physiology in the marine compared to freshwater environment (Browne et al. 2015). Wherever possible, we used gonad and gamete data from a single source, but in numerous cases, data had to be combined from separate studies. To avoid

potential biases by using ratios as explanatory variables in our analyses (Tomkins and Simmons 2002), we converted the gonadosomatic index (GSI: testes divided by body mass) to absolute testes mass based on the reported body mass. When gonad sizes were measured at multiple time-points across the season, we used the maximum mean values or those of individuals reported to be in spawning condition.

The male gamete data included total sperm length, sperm number, average-path sperm velocity (VAP) at approximately 10 s postactivation and sperm longevity (i.e., duration of progressive motility). We restricted these data to manually stripped milt samples because this is the most widely used sampling technique in the fish literature (also see Stockley et al. 1996). We found no direct comparison between stripped and natural ejaculates in fishes to compare our data with natural spawning. However, although stripped samples do not necessarily reflect the volume of milt released at spawning, they do represent the amount of stored milt available for ejaculation and are expected to vary more between than within species. Unless total sperm numbers were reported directly, we multiplied mean milt volume by mean sperm concentration. For females, we used data on absolute fecundity (determined by stripping, in rare cases by postmortem removal), because numbers of eggs released per spawning event were not generally available. Yet, Shapiro et al. (1994) found no significant difference between the number of eggs collected by stripping and during natural spawning in the bluehead wrasse (*Thalassoma bifasciatum*). Further, across 11 of the species examined here, data on spawned eggs published elsewhere (Stockley et al. 1996; Leach 1997) were tightly correlated with our data for stripped samples ($r = 0.77$ [0.33–0.90], $t = 3.67$, $P = 0.005$, $\lambda = 0.67$ [0.00–0.99]).

We took several measures to maximize the comparability of fish data because different sampling conditions among studies can introduce noise to the estimates of gamete numbers and physiology. Particularly sperm velocity and longevity are known to vary with the temperature, pH and osmolarity of the spawning environment (Alavi and Cosson 2005, 2006; Browne et al. 2015) and can further be influenced by the ovarian fluid surrounding the eggs (e.g., Turner and Montgomerie 2002; Urbach et al. 2005; Alonzo et al. 2016). Species-specific environmental optima for data collection or procedural differences are a common caveat of comparative studies using literature data, but given the distribution of data sources across the phylogeny and often combining multiple sources per species, the error introduced to trait comparisons is expected to be random rather than systematic. Yet, to minimize such confounding effects, we restricted sperm performance data to those taken at ambient temperature and the lowest osmolarity reported (thereby approximating freshwater conditions). Further, we recorded whether gametes were collected after hormonally enhancing spermiation or ovulation to statistically account for potential effects on gamete numbers. Wherever possible, we used gamete numbers from nonartificially induced treatment groups, or from the control group where fish were subjected to different experimental treatments.

Finally, in both frogs and fishes, we also collected qualitative information on their spawning conditions. For the anurans, this information included the spawning location (aquatic, terrestrial), presence or absence of foam nests, water movement (stagnant, flowing), oviposition substrate (floating, attached to rocks/vegetation, in nest), oviposition type (one large clutch, several small clutches, single eggs, strands), consistency of the egg capsule (fluid, flexible, firm, viscous), and the mating system (single-male or multi-male amplexus) (Byrne et al. 2002; Fei et al. 2009; Anstis 2013; Zeng et al. 2014). In the fishes, we recorded the egg deposition site (into nest, onto plants/rocks, into open water/scattering over substrate), water movement (stagnant, flowing/turbulent), and the mating system (pair spawning, pair spawning with additional males, group spawning), primarily based on Teletchea et al. (2009), Balon (1975), and information retrieved from FishBase (http://www.fishbase.org).

## PHYLOGENIES

We reconstructed the anuran phylogeny based on nine nuclear, mitochondrial, and mitochondrial ribosome genes. The three nuclear genes included the recombination-activating gene 1 (RAG1), rhodopsin (RHOD), and tyrosinase (TYR). The six mitochondrial genes were cytochrome *b* (CYTB), cytochrome oxidase subunit I (COI), NADH dehydrogenase subunits 2 and 4 (ND2 and ND4), and the large and small subunits of the mitochondrial ribosome genes (12S/16S; omitting the adjacent tRNAs that were difficult to align and represented only a small amount of data). For GenBank accession numbers see Suppl. Data File S2. We aligned the sequences using multi-sequence alignment (MUSCLE) in MEGA v.6.0.6 (Tamura et al. 2013). The best nucleotide substitution models, determined in jModelTest v.2.1.7 (Darriba et al. 2012) based on the Akaike Information Criterion, was GTR+Γ+I for all genes except RHOD, for which HKY+Γ+I had stronger support. We used the same procedure for the fish phylogeny, using the sequences of the RAG1, RHOD, CYTB, COI, and 16S genes, respectively, and GTR+Γ+I as the best substitution model for all genes (GenBank accession numbers in Suppl. Data File S2).

Using BEAUTi and BEAST v.1.8.3 (Drummond et al. 2012), we then constructed both phylogenies with unlinked substitution models, a relaxed uncorrelated log-normal clock and a Yule speciation process. We omitted time calibration due to a lack of fossil dates. Using the BEAST implementation in the CIPRES Science Gateway (http://www.phylo.org), we ran the Markov Chain Monte Carlo (MCMC) simulation for 100 million generations while sampling every 10,000$^{th}$ tree. The effective sample size (ESS) values exceeded 200 for all tree statistics in the program Tracer v.1.6.0

**Table 1. Results of phylogenetic general least-squares models explaining variation in male gamete traits across 25 anuran species (all variables log-transformed).**

| Response | Predictors | $r_p$ | $t$ | $P$ | $R^2$ | λ |
|---|---|---|---|---|---|---|
| Total sperm length | **Testes mass** | **0.88 [0.75, 0.93]** | **8.43** | **<0.0001** | 0.82 | <0.001 [0.00, 1.00] |
| | **Body mass** | **–0.79 [–0.88, –0.57]** | **–5.75** | **<0.0001** | | |
| | Egg size | 0.14 [–0.29, 0.50] | 0.63 | 0.54 | | |
| | **Egg number** | **0.54 [0.14, 0.74]** | **2.79** | **0.01** | | |
| Total sperm number | **Testes mass** | **0.70 [0.41, 0.83]** | **4.40** | **0.0002** | 0.78 | <0.001 [0.00, 0.43] |
| | Body mass | –0.16 [–0.52, 0.27] | –0.73 | 0.47 | | |
| | Egg size | 0.26 [–0.17, 0.58] | 1.22 | 0.24 | | |
| | Egg number | –0.01 [–0.41, 0.39] | –0.07 | 0.95 | | |
| Total gamete | **Testes mass** | **0.80 [0.59, 0.89]** | **6.02** | **<0.0001** | 0.83 | <0.001 [0.00, 0.93] |
| investment (*m***s**) | Body mass | –0.40 [–0.66, 0.03] | –1.95 | 0.07 | | |
| | Egg size | 0.28 [–0.16, 0.59] | 1.29 | 0.21 | | |
| | Egg number | 0.12 [–0.30, 0.49] | 0.55 | 0.59 | | |
| Relative gamete | **Testes mass** | **–0.48 [–0.71, –0.07]** | **–2.46** | **0.02** | 0.70 | <0.001 [0.00, 0.36] |
| investment (*m**/*s**) | Body mass | –0.13 [–0.50, 0.29] | –0.60 | 0.55 | | |
| | Egg size | –0.23 [–0.56, 0.20] | –1.08 | 0.29 | | |
| | Egg number | 0.16 [–0.27, 0.51] | 0.71 | 0.48 | | |

**The partial correlation coefficients, $r_p$, and phylogenetic scaling parameters, λ, are presented with their 95% noncentral confidence limits. Stepwise model simplification did not qualitatively change any of these results, but for direct comparison between response variables and with Table 2, the full models are presented.**

(Rambaut et al. 2014), indicating satisfying convergence of the Bayesian chain and adequate model mixing. Finally, we generated maximum clade credibility trees with mean node heights and a 10% burn-in using TreeAnnotator v.1.8.3 (Drummond et al. 2012), presented in Figs. S1 (frogs) and S2 (fishes).

### STATISTICAL ANALYSES

We conducted all statistical analyses on log-transformed data in the R statistical environment version 3.3.1 (R Development Core Team 2016). We accounted for nonindependence of data through shared ancestry using phylogenetic generalized least-squares (PGLS) models as implemented in the R package *caper* (Orme et al. 2012) and our reconstructed phylogenies. Using a maximum-likelihood approach, PGLS models estimate the phylogenetic scaling parameter λ to evaluate the phylogenetic effect on relationships (λ near 0 indicates phylogenetic independence, and λ near 1 complete phylogenetic dependence: Pagel 1999; Freckleton et al. 2002). Throughout this article, λ values and (partial) correlation coefficients *r* (reflecting the strength of relationships; Nakagawa and Cuthill 2007) are presented with their noncentral 95% confidence limits in squared brackets.

To better compare between frogs and fishes the causal relationships among traits of interest, including potential indirect effects, we further performed phylogenetic confirmatory path analyses (von Hardenberg and Gonzalez-Voyer 2013) based on prespecified candidate path models. Using the R package *phylopath* (van der Bijl 2017), we examined the conditional independences of each model using a PGLS approach, ranked all candidate models based on their C-statistic Information Criterion (CICc) and averaged those with ΔCICc ≤ 2 from the top model (for details on the method and a worked example see von Hardenberg and Gonzalez-Voyer 2013).

## Results

### ANURA

Across all 130 anuran species, sperm length increased with relative testes mass (PGLS; testes mass: partial $r$ ($r_p$) = 0.48 [95%CI: 0.33–0.59], $t$ = 6.11, $P < 0.0001$; body mass: $r_p$ = –0.06 [–0.22 to 0.12], $t$ = –0.63, $P$ = 0.53, λ = 0.93 [0.85–0.97]; Fig. S3A). Similarly, species with multi-male amplexus (i.e., more intense sperm competition) had longer sperm than those reported to mate in pairs (PGLS; $F_{1,127} = 21.92$, $P < 0.0001$, controlling for the significant effect of body mass, $F_{1,127} = 11.79$, $P = 0.0008$; λ = 0.92 [0.84–0.97]; Fig. S3B).

We found qualitatively similar results in a reanalysis across those 25 species with all gamete data available, further controlling for egg size and number (of which only egg number had a significant effect; Table 1; Fig. S4). Across the same 25 species, total sperm number and the total gamete investment (*m***s**) also increased with relative testes mass, whereas the relative gamete investment (*m**/*s**) decreased (Table 1; Fig. S4). Both egg size and number had no significant effect on these ejaculate traits (Table 1). That relative testes mass was a stronger predictor of

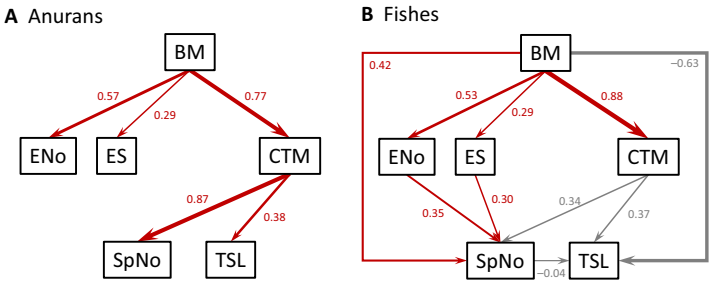


**Figure 1.** **Visual representation of the averaged best-fitting path models (CICc ≤ 2) for the (A) anurans and (B) fishes, respectively. Arrows reflect the direction of the path, and their line width is proportional to their standardized regression coefficients (adjacent to arrows). For red arrows, the 95%CI of the coefficients excluded 0 (i.e., arrows are highly probable), for gray arrows it did not (i.e., arrows are uncertain). The coefficients and their 95%CI are listed in Tables S3 (anurans) and S9 (fishes). The set of candidate path models tested was identical for both taxa (see Fig. S5). BM, body mass; ENo, egg number; ES, egg size; CTM, combined testes mass; SpNo, sperm number; TSL, total sperm length.**

sperm length and number than female gamete investment was further supported by analyses of the nonfoamy aquatic spawners alone (Table S1; permitting more direct comparisons with the fishes) and by phylogenetic confirmatory path analyses (Fig. 1A; Tables S2 and S3) based on 24 prespecified path models (see directed acyclic graphs in Fig. S5).

Next, we examined the relative strength of effects between sperm competition and spawning conditions on variation in sperm length and number in a series of PGLS analyses with testes mass, body mass, and a focal categorical variable as predictors (full details of all analyses in Table S4). We found sperm to be longer in terrestrial than in aquatic breeders ($F_{1,126} = 12.68$, $P = 0.0005$) and, among the latter, foam-nesters had shorter sperm than nonfoamy aquatic breeders ($F_{1,99} = 8.05$, $P = 0.006$). Among the nonfoamy aquatic breeders, sperm length was not significantly affected by the spawning substrates, level of water movement, mode of egg release, or egg-capsule consistency (all $P \geq 0.27$). In all these analyses, however, relative testes mass had a strong effect (all $P \leq 0.0003$). For sperm number, there was only a marginally significant effect of the oviposition substrate, with substrate spawners releasing more sperm than those with floating clutches ($F_{1,17} = 5.05$, $P = 0.04$; all others: $P > 0.28$), whereas relative testes mass again had the strongest effect in all analyses (all $P \leq 0.02$; Table S5).

To better understand potential functional consequences of sperm length variation, we further examined the absolute and relative lengths of the sperm head (carrying the nucleus) and sperm tail (generating propulsion). A relatively longer tail is predicted to generate greater propulsion (given the necessary energy) and has been linked to faster swimming in comparative studies across diverse taxa (Fitzpatrick et al. 2009; Lüpold et al. 2009a; Tourmente et al. 2011). Focusing on sperm competition as the dominating selective force on sperm morphology, we found both sperm head and flagellum length, but also the flagellum/head ratio, to increase with relative testes mass (PGLS: $r_p = 0.20$, $P = 0.03$; Table S6). The result of the flagellum/head ratio was further confirmed by the negatively allometric relationship (i.e., slope $< 1$) between sperm head and flagellum length ($N = 126$, phylogenetic reduced major-axis slope = 0.61 [0.52–0.70], $P < 0.0001$, $\lambda = 0.94$, using R package *phytools*, Revell 2012).

Finally, we examined the effects of the spawning environment on female gametes across 99 species. PGLS analyses controlling for female size ($P \leq 0.0008$) indicated that terrestrial breeders deposit larger but fewer eggs than aquatic breeders ($F_{1,97} \geq 5.45$, $P \leq 0.02$; Table S7; Fig. S6). The same was true for females spawning in flowing compared to stagnant water among the nonfoamy aquatic breeders ($F_{1,61} \geq 6.18$, $P \leq 0.02$; Table S7; Fig. S6). Eggs also tended to be relatively larger if they had a firm or fluid rather than a flexible capsule (overall effect: $F_{3,59} = 4.23$, $P = 0.009$; Table S7; Fig. S6), but no other spawning parameter significantly affected egg size or number (all $P \geq 0.26$; Table S7).

## FISHES

Across the fishes, sperm length was not significantly dependent on relative testes mass (PGLS, $N = 46$; testes mass: $r = 0.21$ [–0.09 to 0.45], $t = 1.37$, $P = 0.18$; body mass: $r = -0.22$ [–0.47 to –0.08], $t = -1.49$, $P = 0.14$, $\lambda = 0.40$ [0.00–0.80]), but it increased (though nonsignificantly) from paired to communal spawners (mating system: $F_{2,36} = 2.78$, $P = 0.08$; body mass: $F_{1,36} = 0.15$, $P = 0.70$; $\lambda = 0.27$). By contrast, when additionally accounting for the positive effect of hormonal treatment ($r = 0.36$ [0.07–0.57], $t = 2.52$, $P = 0.02$), sperm number showed a weak positive relationship with relative testes mass (PGLS, $N = 46$; testes mass: $r = 0.33$ [0.04–0.55], $t = 2.27$, $P = 0.03$; body mass ($r = 0.20$ [–0.10 to 0.45], $t = 2.32$, $P = 0.19$, $\lambda < 0.001$ [0.00–0.41]), but it did not differ between mating systems (PGLS, mating system: $F_{2,31} = 1.95$, $P = 0.16$; body mass: $F_{1,31} = 67.30$, $P < 0.0001$; hormonal induction: $F_{2,31} = 0.65$, $P = 0.47$; $\lambda < 0.001$). (Note that hormonal induction had no significant effect in any further analysis and will, for simplicity, no longer be listed

**Table 2. Results of phylogenetic general least-squares models explaining variation in male gamete investment across 34 fish species (all variables log-transformed).**

| Response | Predictors | $r_p$ | $t$ | $P$ | $R^2$ | λ |
|---|---|---|---|---|---|---|
| Total sperm length[1] | Testes mass | 0.30 [–0.06, 0.56] | 1.68 | 0.10 | 0.11 | 0.24 [0.00–0.76] |
| | Body mass | –0.27 [–0.54, 0.09] | –1.51 | 0.14 | | |
| | Egg size | –0.10 [–0.42, 0.26] | –0.53 | 0.60 | | |
| | Egg number | 0.06 [–0.29, 0.39] | 0.32 | 0.75 | | |
| Total sperm number | Testes mass | 0.08 [–0.27, 0.41] | 0.43 | 0.67 | 0.79 | <0.001 [0.00–0.59] |
| | Body mass | 0.33 [–0.02, 0.59] | 1.91 | 0.07 | | |
| | **Egg size** | **0.38 [0.03, 0.62]** | **2.23** | **0.03** | | |
| | **Egg number** | **0.46 [0.12, 0.67]** | **2.75** | **0.01** | | |
| Total gamete | Testes mass | 0.17 [–0.19, 0.48] | 0.95 | 0.35 | 0.76 | <0.001 [0.00–0.69] |
| Investment (*m***s**) | Body mass | 0.24 [–0.12, 0.53] | 1.35 | 0.19 | | |
| | Egg size | 0.33 [–0.03, 0.59] | 1.90 | 0.07 | | |
| | **Egg number** | **0.43 [0.09, 0.65]** | **2.57** | **0.02** | | |
| Relative gamete | Testes mass | 0.03 [–0.32, 0.37] | 0.16 | 0.87 | 0.76 | <0.001 [0.00–0.50] |
| Investment (*m**/*s**) | **Body mass** | **–0.40 [–0.63, –0.05]** | **–2.32** | **0.03** | | |
| | **Egg size** | **–0.40 [–0.63, –0.06]** | **–2.37** | **0.02** | | |
| | **Egg number** | **–0.45 [–0.66, –0.11]** | **–2.69** | **0.01** | | |

[1]Note that model simplification did not qualitatively change any of these results, including those for sperm length, with no significant effect of relative testes mass after removing both egg variables ($r_p = 0.29$ [–0.06 to 0.55], $t = 1.70$, $P = 0.10$; multiple $R^2 = 0.09$, $\lambda = 0.14$ [0.00–0.71]). For direct comparison of the effects between response variables and with Table 1, however, full models are presented. The partial correlation coefficients, $r_p$, and phylogenetic scaling parameters, λ, are presented with their 95% noncentral confidence limits. Note that hormonal induction had no significant effect on any analysis involving sperm number (all $P > 0.19$) and is therefore omitted for direct comparison with Table 1.

as a predictor in the following sections). Importantly, although the positive effect of relative testes mass alone on sperm number remained when rerunning the above model on those species with additional information on female gametes ($r = 0.33$ [–0.002 to 0.57], $t = 2.02$, $P = 0.05$), it was replaced entirely by the positive effects of both egg size and number when these variables were included in the model (Table 2). Female gamete investment (particularly egg number), but not relative testes mass, also affected *m***s** positively and *m**/*s** negatively, whereas sperm length was not associated with any predictor in the model, even after stepwise model simplification (Table 2; Fig. S7). We again confirmed these effects in phylogenetic path analyses (Fig. 1B; Tables S8 and S9), in which we tested the same 24 prespecified path models as for the anurans (Fig. S5), thereby highlighting the key differences between the two taxa.

To better understand the links between male and female gamete investment, we further examined possible influences of the spawning conditions. Egg number decreased with egg size but increased with female size (PGLS, $N = 41$; egg size: $r = –0.52$ [–0.69 to –0.26], $t = –3.80$, $P = 0.0005$; female body mass: $r = 0.82$ [0.71–0.89], $t = 8.98$, $P < 0.0001$, $\lambda = 0.74$ [0.27–0.91]). Controlling for the significant effect of female body mass in PGLS analyses ($F_{1,32} \geq 17.30$, $P < 0.0002$, $\lambda \geq 0.76$), there was no difference in egg size or number between species spawning in stagnant or turbulent water, respectively ($F_{1,32} \leq 1.07$, $P \geq 0.31$), but the spawning location had a significant effect ($F_{2,32} \geq 6.99$, $P \leq 0.003$; Fig. 2). Overall, nest builders produced fewer but larger eggs compared to species that either deposit eggs onto plants or rocks, or that scatter their gametes in the open water or over the substrate (Fig. 2).

Sperm length did not vary significantly with any of these spawning conditions ($F_{1\text{-}2,34} < 0.74$, $P > 0.39$, $\lambda < 0.001$). However, species spawning in turbulent water released more sperm for their body size than those spawning in stagnant water (water movement: $F_{1,33} = 5.38$, $P = 0.03$, $\lambda < 0.001$; body mass: $F_{1,33} = 77.37$, $P < 0.0001$; Fig. 3A), but the same model revealed no significant effect of the spawning location itself ($F_{2,33} = 0.42$, $P = 0.66$).

Finally, since available models on ejaculate evolution of external fertilizers are based not only on the investment in sperm size and number but primarily on the links between sperm length, velocity, and longevity (see Introduction), we examined across our sample of species the correlations of sperm performance with sperm length and number, but also with proxies of potential selective forces such as sperm competition or sperm limitation. The full statistics of these PGLS analyses are listed in Table S6. In brief, sperm velocity increased with sperm length ($r = 0.68$, $P < 0.0001$; Fig. S8A) but did not covary with sperm longevity or body size-controlled sperm number ($|r| \leq 0.21$, $P \geq 0.25$). By contrast, sperm longevity showed a positively quadratic relationship

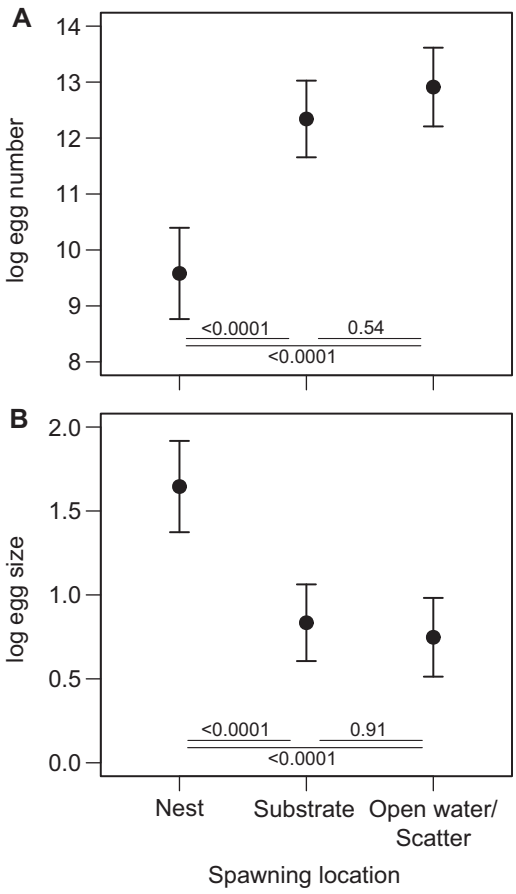


**Figure 2.** Least-squares means with 95% confidence intervals of (A) egg number and (B) egg size between nest brooders ($N = 10$), substrate choosers ($N = 17$), and open-water spawners/substrate scatterers ($N = 11$) in the fishes, after controlling for female body mass. *P*-values of Tukey HSD tests indicate the statistical significance of pairwise comparisons. The analyses were conducted on log-transformed data, but the results were back-transformed for visualization.

with sperm length ($r = 0.42$, $P = 0.008$; Fig. S8B), and was inversely related with relative sperm number ($r = –0.32$, $P = 0.03$; Fig. S8C). Further, sperm velocity was independent of relative testes mass and egg size or number ($r \leq 0.23$, $P \geq 0.27$), whereas sperm longevity was not significantly correlated with female gamete investments ($|r| \leq 0.28$, $P \geq 0.10$) but declined with relative testes mass ($r = –0.44$, $P = 0.007$; Fig. S8D). Finally, in PGLS models examining both the spawning site and water turbulence, the spawning site had no effect on either sperm performance trait (velocity: $F_{2,22} = 0.45$, $P = 0.64$, $\lambda = 0.41$; longevity: $F_{2,33} = 0.18$, $P = 0.84$, $\lambda = 0.92$), but on average lotic spawners had both slower and shorter-lived sperm compared to lentic spawners (velocity: $F_{1,22} = 5.01$, $P = 0.04$, $\lambda = 0.31$; longevity: $F_{1,33} = 4.42$, $P = 0.04$, $\lambda = 0.91$; Fig. 3).

## Discussion

Our study of gamete investment in externally fertilizing frogs and fish revealed different responses to selection between the two taxa. Across our sample of anurans, sperm length as well as sperm number, both separately and jointly, were influenced strongly by the level of sperm competition, but less so, if at all, by the size and number of eggs or most factors capturing variation in spawning conditions. Across the fishes, however, male gamete investment was more tightly related to egg size and number than to relative testes mass. Yet, irrespective of the main factors influencing sperm investment, increasing overall investment coincided in both taxa with relatively greater variation in sperm number than in sperm length, as revealed by the declining $m^*/s^*$ ratio. In the following, we discuss these results in the context of the taxon-specific reproductive biology.

### ANURANS

At face value, the general macroevolutionary patterns for anuran sperm length in our study are consistent with previous reports of

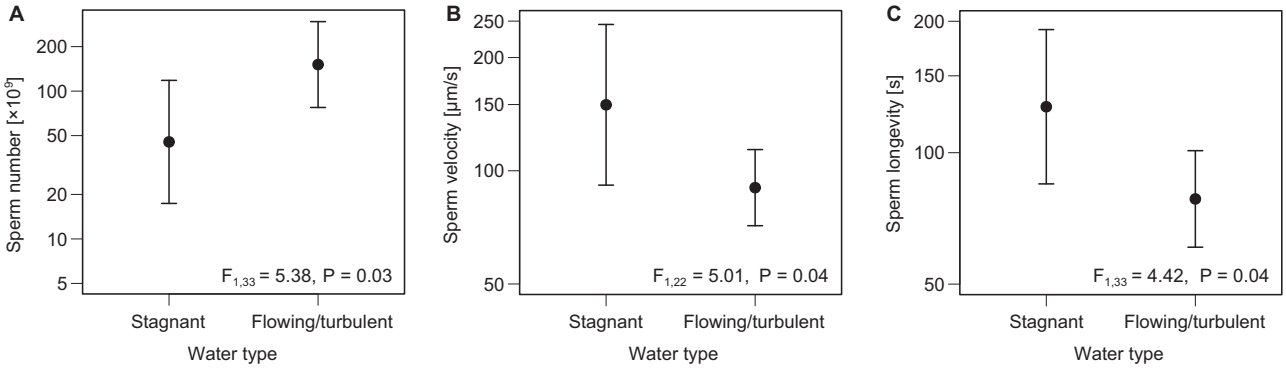


**Figure 3.** Difference in (A) sperm number, (B) sperm velocity, and (C) sperm longevity between species spawning in stagnant or flowing/turbulent water, respectively. The data reflect the least-squares means with 95% confidence intervals retrieved from models with water type and spawning location as predictors (and an additional effect of body mass in A).

sperm head and flagellum elongation in response to intensifying sperm competition in this taxon (Byrne et al. 2003; Zeng et al. 2014). However, we extended these results by also showing positive selection on sperm numbers and, importantly, how both traits jointly (*m***s** and *m**/*s**) respond to selection. That sperm number showed a stronger response than sperm length aligns with theory proposed for situations of raffle-like sperm competition and relatively low sperm density (Parker et al. 2010). While proposed for, and so far examined in, internal fertilizers (Immler et al. 2011; Lüpold and Fitzpatrick 2015), our results indicate that this theory also applies to external fertilizers. Ejaculates released into the environment are vulnerable to sperm dilution or loss. Consequently, even though longer sperm, all else being equal, may increase the competitiveness against rival sperm, mediated by swimming performance or longevity, sperm number likely explains a greater portion of competitive fertilization success in these species.

In addition to positive selection on sperm length by sperm competition, Byrne et al. (2003) reported sperm length to increase with egg size, which they interpreted as an adaptation of sperm to having to penetrate a thicker vestment or jelly capsule when eggs are relatively large, and to traveling a longer distance to the egg nucleus after penetration. Our PGLS and phylogenetic path analyses did not suggest a direct association between male and female gamete sizes but rather separate relations of sperm and eggs with both body size or the spawning environment (Tables 1, S1–S4, S10–S11; Figs. 1A, S9–S10). The body size effect, in males mediated by testes mass (Fig. 1), may reflect some spatial or energetic constraints as total investments in gamete production among anurans appear to increase disproportionately with body size in both males (Lüpold et al. 2017) and females (Monroe et al. 2015; but see Prado and Haddad 2005). Of the environmental effects, the strongest was that of terrestrial versus aquatic breeding. Terrestrial breeders produce relatively larger eggs, which are thought to better provision the tadpoles developing under the unpredictable conditions in terrestrial environments (Bradford 1990). That terrestrial species also exhibit longer sperm may thus be an adaptation to differences in the biochemical composition of the gelatinous egg surroundings in response to terrestrial breeding (e.g., protection against desiccation) rather than to a thicker membrane or jelly coat of larger eggs *per se*. We found no differential sperm length in response to our crude classification of egg capsule consistency, but potential effects of more subtle biochemical differences remain to be explored. Differential selection on sperm form and function by the egg environment may also contribute to the difference in sperm length between foamy and nonfoamy aquatic breeders (also see Muto and Kubota 2013). For example, it has been suggested that foam nests may increase the fertilization efficiency (Byrne et al. 2002; Edwards et al. 2004), but a broad, systematic examination of the consequences of foam, or any variation in its density and viscosity, on sperm performance and gross morphology is currently lacking.

Despite these effects of the spawning environment on variation in ejaculate traits, the vast majority of the variation was explained by relative testes mass in all our analyses of anurans, suggesting that sperm competition probably plays a more important role overall in the evolution of anuran ejaculates than does the spawning environment. Yet, possible links between the two selective pressures are intriguing for further detailed investigation. For example, the lowered risk of sperm loss and heightened sperm longevity and fertilization efficiency through foam nesting has been suggested to intensify sperm competition compared to strictly aquatic spawning (Byrne et al. 2002). Further, a recent study suggests that the repeated evolutionary transition to terrestrial oviposition in hylid and leptodactylid frogs may be linked to a higher chance of hidden amplexus on land than in the water, thereby favoring males that can lower the risk of multi-male spawning through terrestrial mating (Zamudio et al. 2016). It would thus be interesting to disentangle the links between different forms of polyandry (from multi-male amplexus to clutch piracy) and spawning conditions, including the relative timing of gamete release between the sexes, to better understand the fertilization processes and selection on different ejaculate traits.

Finally, we found sperm tail length to increase disproportionately compared to sperm head length with any increase in total sperm length, both based on the allometric exponents and the response of the tail/head ratio to relative testes mass. These results contrast with two previous studies that suggested the opposite (Byrne et al. 2003; Zeng et al. 2014). (Note, however, that the data of both these studies also show considerably greater variation in tail than head length, which would be consistent with our results rather than the opposite as reported.) A relatively longer sperm tail is associated interspecifically with faster sperm swimming in numerous taxa (Gomendio and Roldan 2008; Fitzpatrick et al. 2009; Lüpold et al. 2009a; Tourmente et al. 2011), but data to examine the link between sperm morphology and sperm performance across the anurans are largely lacking. In general, frog sperm swim slowly (Browne et al. 2015), and slower and prolonged swimming increases competitive fertilization success in *C. georgiana* (Dziminski et al. 2009), though with no direct link between sperm performance and morphology (Dziminski et al. 2010). Thus, even if sperm velocity trades off with sperm longevity, it remains unclear as to how these traits covary with sperm morphology across species. Ball and Parker's (1997) continuous fertilization model would predict selection for longer sperm tails (generating greater propulsion) to reduce sperm longevity, but the possible benefits of sperm endurance combined with selection for longer sperm (particularly tails) may also suggest that longer sperm live longer but swim more slowly. If so, the theoretical predictions would not necessarily translate to anuran sperm, which may not be surprising

given their considerably different morphology, metabolism, and movement patterns from fish or other vertebrates (Browne et al. 2015). This ambiguity emphasizes the need for a robust comparative dataset on sperm morphology, velocity, and longevity in anurans to disentangle their covariation, ideally also considering potentially differential adaptations to the spawning conditions.

## FISHES

Similar to anurans, previous comparative studies have indicated a positive link between sperm competition and sperm length across fishes (Balshine et al. 2001; Fitzpatrick et al. 2009; Montgomerie and Fitzpatrick 2009). Positive selection on sperm length is thought to be the result of selection for faster sperm due to their fertilization advantage (Fitzpatrick et al. 2009). Our results confirmed such a link between sperm length and velocity, thereby expanding Fitzpatrick et al.'s (2009) study of cichlids to a broader range of fishes. Unlike the above studies, however, we found no clear positive relationship between sperm length and sperm competition when using residual testes mass as a proxy of sperm competition, but communal spawners tended to have slightly longer sperm than pair breeders. In isolation, our analyses also revealed a positive trend for sperm number, consistent with Stockley et al.'s (1997) findings in a subset of the species studied here. Importantly, however, when examining the effects of egg size and number on male gamete investment simultaneously with a proxy of sperm competition, the effect of the latter was completely overwhelmed by those of female gametes. This was also true for the combined measures of male gamete investments, $m^*s^*$ and $m^*/s^*$, both of which reflected similar interspecific patterns as in the anurans but in response to female gametes rather than sperm competition. At least for our set of species it thus appears that sperm limitation may play at least as important, if not more important, a role in the evolution of fish ejaculates compared to sperm competition.

Further support for the likely importance of sperm limitation through gamete scattering in moving water comes from the contributions of spawning conditions (e.g., water turbulence) to sperm number and sperm physiology, in that species breeding in flowing or turbulent water produced more but slower and shorter-lived sperm. Consistently, sperm longevity (but not sperm velocity) was negatively associated with sperm number. It is thus possible that in moving water sperm swimming speed and longevity used for active sperm swimming toward eggs become relatively less important compared to the increased passive movement of male and female gametes through water currents that may ultimately result in random sperm–egg collisions. If so, the marginal benefits of maximizing sperm number and delivering sperm the closest possible to the egg mass would increasingly outweigh those of producing faster sperm as the spawning environment becomes more dynamic. This begs the question as to how varying degrees of water turbulence would influence the relative importance of sperm number and sperm performance. By measuring sperm velocity under the controlled conditions of a microscope slide and often conducting competitive fertilization trials in a petri dish with standardized sperm numbers, there is a risk of overestimating the role of sperm velocity on competitive fertilization, particularly in species that naturally breed in turbulent water. Considering the effects of water currents and gamete dispersal in the fertilization process might thus be a promising avenue for further examination of the adaptive significance of, and multivariate selection on, ejaculate traits. Comparisons between populations of a given species that occupy different water conditions might be particularly revealing.

The link between sperm and egg quantity has been previously reported for the group-spawning bluehead wrasse (Shapiro et al. 1994) and has been suggested, albeit equivocally, across fish species (Stockley et al. 1996). In our study, egg number was strongly influenced by the spawning conditions, being greatest when eggs are broadcast into the open water and smallest in species that deposit them into a nest. Variation in egg numbers should exert selection on sperm number. Being released in greater numbers, eggs may disperse over a larger volume of water, and so a greater volume and density of sperm may have to be released to locate and fertilize these eggs, given that each sperm can at best swim a few millimeters in its short lifetime (Shapiro et al. 1994; Stockley et al. 1996; Browne et al. 2015). Through the trade-off between egg size and number, the targets for sperm would additionally become smaller, thereby further enhancing selection on sperm number due to the lower probability of sperm–egg encounters (Levitan 1993, 2006; Rahman and Uehara 2004; Macfarlane et al. 2009). Interestingly, however, sperm number covaried positively with both egg number and egg size. The reason for this relationship is less clear. For example, although it is possible that more sperm increase the probability of finding the miniscule micropyle on larger eggs, one would also expect biochemical and physical interactions between sperm and eggs to guide sperm to the micropyle upon the encounter (Robertson 1996). Further, it has been proposed that sperm limitation is more probable in pair-bonding species than when multiple males contribute sperm, and that the evolution of larger eggs is a response to it (i.e., increasing target size; Levitan 1993; Robertson 1996). Consequently, while it may be difficult to clearly separate cause and effect between egg size and sperm number, possible effects of sperm limitation may also become conflated with those of sperm competition. Disentangling these different effects is particularly challenging without detailed knowledge of how males allocate their sperm between avoiding fertilization failure, maximizing competitive fertilization success, and being able to mate repeatedly without depleting sperm reserves. Out of necessity, we relied on stripped samples in our analyses. Similarly, egg data from natural spawning events (rather than our necessary use of total fecundity due to data

availability) would allow us to examine how the release of eggs in clutches, or the egg dispersal in the water, affect the fertilization dynamics and corresponding sperm demands.

## Conclusions

Despite certain limitations given the nature of the data currently available, our simultaneous consideration of multiple ejaculate traits and their interrelationships revealed contrasting patterns of gamete evolution between externally fertilizing frogs and fish. Our results suggest in both taxa that sperm number responds more strongly to selection than sperm length and the spawning environment is likely to play a critical role. Yet, an important difference between the two taxa was that the mating system was the primary selective force on ejaculate traits in the anurans, but egg size and number (and thus possibly sperm limitation) had a relatively stronger effect in the fishes. The contrasting results between the two taxa are likely linked to differential spawning processes and conditions, with anuran sperm swimming for extended periods and competing in a somewhat protected environment of the egg jelly, and fish sperm being released into the water, close to the eggs, where they have to encounter eggs rapidly to avoid dispersal.

Our results indicate that by focusing our attention primarily on sexual selection as a driver of ejaculate evolution we might underestimate the role and importance of other selective processes. We emphasize the value of examining ejaculates as multivariate traits and combining proxies of both sexual and natural selection for a more nuanced understanding of macroevolutionary variation. Clearly, our study barely scratched the surface of the tremendous diversity in reproductive modes and traits in both taxa examined, and the scarce data currently available (relative to the number of species) limit the generalization of our conclusions. Consequently, we look forward to future experimental and comparative studies that will address some of the patterns revealed here more specifically by integrating further sperm functional traits and, ideally, using data from natural breeding events despite their logistic challenges.

### AUTHOR CONTRIBUTIONS

W.B.L. and S.L. designed the study; all authors collected the data; S.L. generated the phylogenies and analyzed the data; W.B.L. and S.L. wrote the paper; all authors edited and approved the final draft.

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## CONFLICT OF INTEREST

The authors declare that they have no conflict of interest.

### DATA ARCHIVING

The doi for our data is Dryad, https://doi.org/10.5061/dryad.t5jp7

### LITERATURE CITED

Alavi, S. M. H., and J. Cosson. 2005. Sperm motility in fishes. I. Effects of temperature and pH: a review. Cell Biol. Int. 29:101–110.

———. 2006. Sperm motility in fishes. (II) Effects of ions and osmolality: a review. Cell Biol. Int. 30:1–14.

Alonzo, S. H., K. A. Stiver, and S. E. Marsh-Rollo. 2016. Ovarian fluid allows directional cryptic female choice despite external fertilization. Nat. Commun. 7:12452.

Anstis, M. 2013. Tadpoles and frogs. New Holland Publishers, London.

Ball, M. A., and G. A. Parker. 1996. Sperm competition games: external fertilization and "adaptive" infertility. J. Theor. Biol. 180:141–150.

———. 1997. Sperm competition games: inter- and intra-species results of a continuous external fertilization model. J. Theor. Biol. 186:459–466.

Balon, E. K. 1975. Reproductive guilds of fishes: a proposal and definition. J. Fish Res. Board Canada 32:821–864.

Balshine, S., B. J. Leach, F. Neat, N. Y. Werner, and R. Montgomerie. 2001. Sperm size of African cichlids in relation to sperm competition. Behav. Ecol. 12:726–731.

Birkhead, T. R., D. J. Hosken, and S. Pitnick. 2009. Sperm biology: an evolutionary perspective. Academic Press, San Diego.

Birkhead, T. R., and A. P. Møller. 1998. Sperm competition and sexual selection. Academic Press, San Diego.

Bjork, A., and S. Pitnick. 2006. Intensity of sexual selection along the anisogamy-isogamy continuum. Nature 441:742–745.

Bradford, D. F. 1990. Incubation time and rate of embryonic development in amphibians: the influence of ovum size, temperature, and reproductive mode. Physiol. Zool. 63:1157–1180.

Briskie, J. V, R. Montgomerie, and T. R. Birkhead. 1997. The evolution of sperm size in birds. Evolution 51:937–945.

Browne, R. K., S. A. Kaurova, V. K. Uteshev, N. V. Shishova, D. McGinnity, C. R. Figiel, N. Mansour, D. Agnew, M. Wu, E. N. Gakhova, et al. 2015. Sperm motility of externally fertilizing fish and amphibians. Theriogenology 83:1–13.

Byrne, P. G. 2004. Male sperm expenditure under sperm competition risk and intensity in quacking frogs. Behav. Ecol. 15:857–863.

Byrne, P. G., J. D. Roberts, and L. W. Simmons. 2002. Sperm competition selects for increased testes mass in Australian frogs. J. Evol. Biol. 15:347–355.

Byrne, P. G., L. W. Simmons, and J. D. Roberts. 2003. Sperm competition and the evolution of gamete morphology in frogs. Proc. R Soc. B 270:2079–2086.

Crean, A. J., and D. J. Marshall. 2008. Gamete plasticity in a broadcast spawning marine invertebrate. Proc. Natl. Acad. Sci. USA 105:13508–13513.

Darriba, D., G. L. Taboada, R. Doallo, and D. Posada. 2012. jModelTest 2: more models, new heuristics and parallel computing. Nat. Methods 9:772.

Denny, M. W., and M. F. Shibata. 1989. Consequences of surf-zone turbulence for settlement and external fertilization. Am. Nat. 134:859–889.

Drummond, A. J., M. A. Suchard, D. Xie, and A. Rambaut. 2012. Bayesian phylogenetics with BEAUti and the BEAST 1.7. Mol. Biol. Evol. 29:1969–1973.

Dziminski, M. A., J. D. Roberts, M. Beveridge, and L. W. Simmons. 2009. Sperm competitiveness in frogs: slow and steady wins the race. Proc. R Soc. B 276:3955–3961.

Dziminski, M. A., J. D. Roberts, and L. W. Simmons. 2010. Sperm morphology, motility and fertilisation capacity in the myobatrachid frog *Crinia georgiana*. Reprod. Fertil. Dev. 22:516–522.

Edwards, D. L. A., M. J. B. Mahony, and J. B. Clulow. 2004. Effect of sperm concentration, medium osmolality and oocyte storage on artificial fertilisation success in a myobatrachid frog (*Limnodynastes tasmaniensis*). Reprod. Fertil. Dev. 16:347–354.

Fei, L., S. Q. Hu, C. Y. Ye, and Y. Z. Huang. 2009. Fauna Sinica. Amphibia (vol. 3), Anura Ranidae. Science Press, Bejing.

Fitzpatrick, J. L., and S. Lüpold. 2014. Sexual selection and the evolution of sperm quality. Mol. Hum. Reprod. 20:1180–1189.

Fitzpatrick, J. L., R. Montgomerie, J. K. Desjardins, K. A. Stiver, N. Kolm, and S. Balshine. 2009. Female promiscuity promotes the evolution of faster sperm in cichlid fishes. Proc. Natl. Acad. Sci. USA 106:1128–1132.

Franzén, Å. 1970. Phylogenetic aspects of the morphology of spermatozoa and spermiogenesis. Pp. 29–45 *in* B. Baccetti, ed. Comparative spermatology. Academic Press, New York.

Freckleton, R. P., P. H. Harvey, and M. Pagel. 2002. Phylogenetic analysis and comparative data: a test and review of evidence. Am. Nat. 160:712–726.

Gage, M. J. G. 1994. Associations between body size, mating pattern, testis size and sperm lengths across butterflies. Proc. R Soc. B 258:247–254.

Gomendio, M., and E. R. S. Roldan. 2008. Implications of diversity in sperm size and function for sperm competition and fertility. Int. J. Dev. Biol. 52:439–447.

Gómez Montoto, L., C. Magaña, M. Tourmente, J. Martín-Coello, C. Crespo, J. J. Luque-Larena, M. Gomendio, and E. R. S. Roldan. 2011. Sperm competition, sperm numbers and sperm quality in muroid rodents. PLoS One 6:e18173.

Hettyey, A., and J. D. Roberts. 2007. Sperm traits in the quacking frog (*Crinia georgiana*), a species with plastic alternative mating tactics. Behav. Ecol. Sociobiol. 61:1303–1310.

Immler, S., S. Pitnick, G. A. Parker, K. L. Durrant, S. Lüpold, S. Calhim, and T. R. Birkhead. 2011. Resolving variation in the reproductive tradeoff between sperm size and number. Proc. Natl. Acad. Sci. USA 108:5325–5330.

Jin, L., Z. P. Mi, and W. B. Liao. 2016a. Altitudinal variation in male reproductive investment in a polyandrous frog species (*Hyla gongshanensis jingdongensis*). Anim. Biol. 66:289–303.

Jin, L., S. N. Yang, W. B. Liao, and S. Lüpold. 2016b. Altitude underlies variation in the mating system, somatic condition, and investment in reproductive traits in male Asian grass frogs (*Fejervarya limnocharis*). Behav. Ecol. Sociobiol. 70:1197–1208.

Katz, D. F., and E. Z. Drobnis. 1990. Analysis and interpretation of the forces generated by spermatozoa. Pp. 125–137 *in* B. D. Bavister, J. Cummis, and E. R. S. Roldan, eds. Fertilization in mammals. Serano Symposium, Norwell, Massachusetts.

Leach, B. J. 1997. Competition and the evolution of sperm characteristics in Pacific salmon (*Oncorhynchus* spp). Queen's University, Kingston, Ontario, Canada.

Levitan, D. R. 1993. The importance of sperm limitation to the evolution of egg size in marine invertebrates. Am. Nat. 141:517–536.

———. 2006. The relationship between egg size and fertilization success in broadcast-spawning marine invertebrates. Integr. Comp. Biol. 46:298–311.

Liao, W. B., S. L. Lou, Y. Zeng, and A. Kotrschal. 2016. Large brains, small guts: the expensive tissue hypothesis supported within anurans. Am. Nat. 188:693–700.

Lüpold, S. 2013. Ejaculate quality and constraints in relation to sperm competition levels among eutherian mammals. Evolution 67:3052–3060.

Lüpold, S., S. Calhim, S. Immler, and T. R. Birkhead. 2009a. Sperm morphology and sperm velocity in passerine birds. Proc. R Soc. B 276:1175–1181.

Lüpold, S., and J. L. Fitzpatrick. 2015. Sperm number trumps sperm size in mammalian ejaculate evolution. Proc. R Soc. B 282:20152122.

Lüpold, S., L. Jin, and W. B. Liao. 2017. Population density and structure drive differential investment in pre- and postmating sexual traits in frogs. Evolution 71:1686–1699.

Lüpold, S., G. M. Linz, and T. R. Birkhead. 2009b. Sperm design and variation in the New World blackbirds (Icteridae). Behav. Ecol. Sociobiol. 63:899–909.

Lüpold, S., G. M. Linz, J. W. Rivers, D. F. Westneat, and T. R. Birkhead. 2009c. Sperm competition selects beyond relative testes size in birds. Evolution 63:391–402.

Lüpold, S., M. K. Manier, K. S. Berben, K. J. Smith, B. D. Daley, S. H. Buckley, J. M. Belote, and S. Pitnick. 2012. How multivariate ejaculate traits determine competitive fertilization success in *Drosophila melanogaster*. Curr. Biol. 22:1667–1672.

Lüpold, S., M. K. Manier, N. Puniamoorthy, C. Schoff, W. T. Starmer, S. H. B. Luepold, J. M. Belote, and S. Pitnick. 2016. How sexual selection can drive the evolution of costly sperm ornamentation. Nature 533:535–538.

Macfarlane, C. P., D. J. Hoysak, N. R. Liley, and M. J. G. Gage. 2009. In vitro fertilization experiments using sockeye salmon reveal that bigger eggs are more fertilizable under sperm limitation. Proc. R Soc. B 276:2503–2507.

Manier, M. K., S. Lüpold, J. M. Belote, W. T. Starmer, K. S. Berben, O. Ala-Honkola, W. F. Collins, and S. Pitnick. 2013. Postcopulatory sexual selection generates speciation phenotypes in *Drosophila*. Curr. Biol. 23:1853–1862.

Miller, G. T., and S. Pitnick. 2002. Sperm-female coevolution in *Drosophila*. Science 298:1230–1233.

Monroe, M. J., S. H. South, and S. H. Alonzo. 2015. The evolution of fecundity is associated with female body size but not female-biased sexual size dimorphism among frogs. J. Evol. Biol. 28:1793–1803.

Montgomerie, R., and J. L. Fitzpatrick. 2009. Testis size, sperm size and sperm competition. Pp. 1–53 *in* B. G. M. Jamieson, ed. Reproductive biology and phylogeny of fishes. Science Publishers, Enfield, New Hampshire, USA.

Muto, K., and H. Y. Kubota. 2013. Ultrastructure and motility of the spermatozoa of *Polypedates leucomystax* (Amphibia, Anura, Rhacophoridae). Cytoskeleton 70:121–133.

Nakagawa, S., and I. C. Cuthill. 2007. Effect size, confidence interval and statistical significance: a practical guide for biologists. Biol. Rev. 82:591–605.

Orme, C. D. L., R. P. Freckleton, G. H. Thomas, T. Petzoldt, and S. A. Fritz. 2012. caper: comparative analyses of phylogenetics and evolution in R (http://R-Forge.R-project.org/projects/caper/).

Pagel, M. 1999. Inferring the historical patterns of biological evolution. Nature 401:877–884.

Parker, G. A. 1993. Sperm competition games: sperm size and sperm number under adult control. Proc. R. Soc. B 253:245–254.

Parker, G. A. 1982. Why are there so many tiny sperm? Sperm competition and the maintenance of two sexes. J. Theor. Biol. 96:281–294.

———. 2016. The evolution of expenditure on testes. J. Zool. 298:3–19.

Parker, G. A., and M. E. Begon. 1993. Sperm competition games: sperm size and sperm number under gametic control. Proc. R Soc. B 253:255–262.

Parker, G. A., S. Immler, S. Pitnick, and T. R. Birkhead. 2010. Sperm competition games: sperm size (mass) and number under raffle and displacement, and the evolution of $P_2$. J. Theor. Biol. 264:1003–1023.

Pennington, J. T. 1985. The ecology of fertilization of echinoid eggs: the consequences of sperm dilution, adult aggregation, and synchronous spawning. Biol. Bull. 169:115–120.

Pitnick, S. 1996. Investment in testes and the cost of making long sperm in *Drosophila*. Am. Nat. 148:57–80.

Pitnick, S., D. J. Hosken, and T. R. Birkhead. 2009. Sperm morphological diversity. Pp. 69–149 *in* T. R. Birkhead, D. J. Hosken, and S. Pitnick, eds. Sperm biology: An evolutionary perspective. Academic Press, San Diego.

Pizzari, T., and G. A. Parker. 2009. Sperm competition and sperm phenotype. Pp. 207–245 *in* T. R. Birkhead, D. J. Hosken, and S. Pitnick, eds. Sperm biology: An evolutionary perspective. Academic Press, San Diego.

Prado, C. P. A., and C. F. B. Haddad. 2005. Size-fecundity relationships and reproductive investment in female frogs in the Pantanal, south-western Brazil. Herpetol. J. 15:181–189.

Rahman, S. M., and T. Uehara. 2004. Effects of egg size on fertilization and embryonic development of sibling tropical sea urchins (Genus, *Echinometra*). ScienceAsia 30:75–80.

Rambaut, A., M. A. Suchard, D. Xie, and A. J. Drummond. 2014. Tracer v1.6, available from http://beast.bio.ed.ac.uk/Tracer.

Revell, L. J. 2012. phytools: An R package for phylogenetic comparative biology (and other things). Methods Ecol. Evol. 3:217–223.

Roberts, J. D., and P. G. Byrne. 2011. Polyandry, sperm competition, and the evolution of anuran amphibians. Adv. Study Behav. 43:1–53.

Robertson, D. R. 1996. Egg size in relation to fertilization dynamics in free-spawning tropical reef fishes. Oecologia 108:95–104.

Shapiro, D. Y., A. Marconato, and T. Yoshikawa. 1994. Sperm economy in a coral reef fish, *Thalassoma bifasciatum*. Ecology 75:1334–1344.

Simmons, L. W., and J. L. Fitzpatrick. 2012. Sperm wars and the evolution of male fertility. Reproduction 144:519–534.

Snook, R. R. 2005. Sperm in competition: not playing by the numbers. Trends Ecol. Evol. 20:46–53.

Stockley, P., M. J. G. Gage, G. A. Parker, and A. P. Møller. 1996. Female reproductive biology and the coevolution of ejaculate characteristics in fish. Proc. R. Soc. London B 263:451–458.

Stockley, P., M. J. G. Gage, G. A. Parker, and A. P. Møller. 1997. Sperm competition in fishes: the evolution of testis size and ejaculate characteristics. Am. Nat. 149:933–954.

Tamura, K., G. Stecher, D. Peterson, A. Filipski, and S. Kumar. 2013. MEGA6: molecular evolutionary genetics analysis version 6.0. Mol. Biol. Evol. 30:2725–2729.

Teletchea, F., A. Fostier, E. Kamler, J.-N. Gardeur, P.-Y. Le Bail, B. Jalabert, and P. Fontaine. 2009. Comparative analysis of reproductive traits in 65 freshwater fish species: application to the domestication of new fish species. Rev. Fish Biol. Fish. 19:403–430.

Tomkins, J. L., and L. W. Simmons. 2002. Measuring relative investment: a case study of testes investment in species with alternative male reproductive tactics. Anim. Behav. 63:1009–1016.

Tourmente, M., M. Gomendio, and E. R. S. Roldan. 2011. Sperm competition and the evolution of sperm design in mammals. BMC Evol. Biol. 11:12.

Turner, E., and R. Montgomerie. 2002. Ovarian fluid enhances sperm movement in Arctic charr. J. Fish Biol. 60:1570–1579.

Urbach, D., I. Folstad, and G. Rudolfsen. 2005. Effects of ovarian fluid on sperm velocity in Arctic charr (*Salvelinus alpinus*). Behav. Ecol. Sociobiol. 57:438–444.

van der Bijl, W. 2017. phylopath: perform phylogenetic path analysis (https://cran.r-project.org/web/packages/phylopath).

von Hardenberg, A., and A. Gonzalez-Voyer. 2013. Disentangling evolutionary cause-effect relationships with phylogenetic confirmatory path analysis. Evolution 67:378–387.

Zamudio, K. R., R. C. Bell, R. C. Nali, C. F. B. Haddad, and C. P. A. Prado. 2016. Polyandry, predation, and the evolution of frog reproductive modes. Am. Nat. 188:S41–S61.

Zeng, Y., S. L. Lou, W. B. Liao, and R. Jehle. 2014. Evolution of sperm morphology in anurans: insights into the roles of mating system and spawning location. BMC Evol. Biol. 14:104.

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## *Supporting Information*

Additional Supporting Information may be found in the online version of this article at the publisher's website:

**Figure S1.** Phylogenetic reconstruction for the 130 anurans used in this study.
**Figure S2.** Phylogenetic reconstruction for the 57 fishes used in this study.
**Figure S3.** Relationship of total sperm length with relative testes mass or mating system across 130 anuran species.
**Figure S4.** Relationships of total sperm length, sperm number, total gamete investment ($m^*s^*$) and relative gamete investment ($m^*/s^*$) with testes mass across 25 anuran species.
**Figure S5.** Directed acyclic graphs representing 24 candidate models that were compared to disentangle the relationships between six traits in anurans and fish through phylogenetic confirmatory path analyses and multi-model inference.
**Figure S6.** Effects of spawning location, water type and egg capsule consistency on egg size and number in anurans.
**Figure S7.** Relationships of total sperm length, sperm number, total gamete investment ($m^*s^*$) and relative gamete investment ($m^*/s^*$) with egg number across 34 fish species.
**Figure S8.** Relationships of sperm velocity and sperm longevity with sperm length, and of sperm longevity with sperm number and relative testes mass, respectively.
**Figure S9.** Directed acyclic graphs representing 12 candidate models that were compared to disentangle the relationships between five traits in anurans (non-foamy aquatic breeders) through phylogenetic confirmatory path analyses and multi-model inference.
**Figure S10.** Visual representation of the averaged best-fitting path models explaining variation in sperm length in the anurans (non-foamy aquatic breeders) based on the candidate models depicted in Fig. S9.
**Table S1.** Results of phylogenetic generalized least-squares analyses explaining variation in sperm length and number across anurans with non-foamy aquatic spawning.
**Table S2.** Results of the phylogenetic path analyses in the anurans.
**Table S3.** Path statistics of the best-fitting model of Table S2.
**Table S4.** Results of phylogenetic analyses of covariance examining the effects of different spawning conditions on sperm length in the anurans.
**Table S5.** Results of phylogenetic analyses of covariance examining the effects of different spawning conditions on sperm number in the anurans.
**Table S6.** Results of phylogenetic generalized least-squares analyses explaining variation in the lengths of the sperm components across the anuran species.
**Table S7.** Results of phylogenetic analyses of covariance examining the effects of different spawning conditions on egg size and number in the anurans.
**Table S8.** Results of the phylogenetic path analyses in the fishes.
**Table S9.** Path statistics of the best-fitting models in Table S8.
**Table S10.** Results of phylogenetic generalized least-squares analyses explaining variation in either sperm velocity or sperm longevity across the fishes.
**Table S11.** Results of the phylogenetic path analyses examining different trajectories through which female gametes and sperm competition explain variation in sperm length in the anurans.
**Table S12.** Path statistics of the best-fitting models in Table S11.
**Data File S1.** Data on ejaculate traits, female gametes, body size and spawning conditions for all frog and fish species of this study.
**Data File S2.** Genbank accession numbers of all frog and fish species used in this study.